



## **A year (2014-2015) of plants in Proteomics journal. Progress in wet and dry methodologies, moving from protein catalogs, and the view of classic plant biochemists**

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**A year (2014-2015) of plants in *Proteomics* journal. Progress in wet and dry methodologies, moving from protein catalogs, and the view of classic plant biochemists**

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# Abstract

The present review is an update of the previous one published in *Proteomics 2015 Reviews* special issue [1] covering the 2014-July 2015 period. It has been written on the bases of the publications that appeared in *Proteomics* journal during that period and the most relevant ones that have been published in other high-impact journals. Methodological advances and the contribution of the field to the knowledge of plant biology processes and its translation to agroforestry and environmental sectors will be discussed. This review has been organized in four blocks, with a starting general introduction (literature survey) followed by sections focusing on the methodology (*in vitro*, *in vivo*, wet and dry), proteomics integration with other approaches (systems biology and proteogenomics), biological information, and knowledge (cell communication, receptors and signaling), ending with a brief mention of some other biological and translational topics to which proteomics has made some contribution.

## INTRODUCTION

The topic of plant proteomics was reviewed in 2015 on the basis of the articles published in *Proteomics* journal (from now just *Proteomics*, written in italics) between 2001, when the first issue appeared, and May 2014 [1]. The present review is an update covering the January 2014-July 2015 period in which methodological advances and the contribution of the field to the plant biology knowledge and its translation to agroforestry and environmental sectors will be discussed. It is not intended to be repetitive considering recent reviews but aiming to get to the reader for a deeper discussion of key topics. It was written to be included in the 2016 *Proteomics Review Special Issue*, since it is the last one of the series edited by Prof. Dunn, the editor in chief of *Proteomics* since it appeared and who recently retired. It is a matter of justice to recognize the enormous effort of Prof. Dunn in establishing and consolidating *Proteomics* as a leader journal as well as his scientific contribution to the field. The corresponding author, in particular, and the plant community, in general, should acknowledge the personal consideration and the support he always gave to us reflected, just as an example, in his input to the International Plant Proteomics Organization (INPPO; <http://www.inppo.com/>) society [2], even though plant studies are not within his field of investigation. He, better than nobody else, has been an actor, director, and observer of the evolution of Proteomic Science [3].

According to PubMed, during the period of 2014-July 2015 *Proteomics* published 600 papers distributed in 40 issues, either regular or special including *Reviews 2014 and 2015 Tissue Proteomics and Imaging Mass Spectrometry*, *Top-Down Proteomics*, *Focus on Biomedical Research Trends*, *Proteomics in Chromatin Biology and Epigenetics*, *Proteogenomics*, *Signal Transduction*, *Proteomics Data Visualization*, *Plant-based Foods: Seed, Nutrition and Human Health*, *Virology meets*

79 *Proteomics, Views on Protein N-terminal Biology, and Protein Structure*. These thematic issues  
80 perfectly represented the most recent technical advances, the frontier of knowledge in biology, and the  
81 interest in translational biomedicine. Of 592 papers published in *Proteomics*, 133 were selected for  
82 preparing this review as they dealt with plants (33 original papers, 16 reviews, and 1 meeting report,  
83 representing around 10 % of the total papers released by the journal) or presented the state of the art  
84 of the field from a biological, methodological or translational point of view.

85 After a PubMed search the terms proteomics or proteome in the title/abstract returned 8553  
86 hits. Compared with the other -omics approaches, this figure was slightly or much higher than  
87 transcriptomics/transcriptome (7728) or metabolomics/metabolome (3064) searches, respectively.  
88 While looking for the combined use of the different omics approaches, PubMed provided 52  
89 proteomics, transcriptomics, and metabolomics reports, as well as 113 proteomics and  
90 transcriptomics, and 56 proteomics and metabolomics papers. The integration of the different -omics  
91 approaches will be discussed in a further section.

92 In the surveyed period, plants continued to be underrepresented in the current proteomics  
93 literature, but significantly increasing, with 683 references in PubMed (almost 15% of the total) while  
94 for humans, just as a comparative example, the figure was 2795, with quite a few related to the study  
95 of cancer, as well as vascular and other diseases [4]. Despite the claimed general worry about food  
96 supply, environmental concerns, and climate change, these issues have not been properly reflected in  
97 research funding policy in most of the western countries and even less in developing nations. This  
98 issue is very well discussed by Donald Kennedy (President Emeritus at Stanford University, and a  
99 former editor-in-chief of Science) in Science's editorial "Building Agricultural Research" [5]. Human  
100 welfare depends on well-connected health and food supply [6-8]. Famine is the major cause of  
101 mortality on a worldwide basis, and increasing nutritional value of primary crops would drastically  
102 reduce health problems in the less developed countries [9]. The situation seems to be changing, but  
103 very slowly; at HUPO (<https://www.hupo.org/>) or general proteomics meetings such as European  
104 EuPA (<http://www.eupa.org/>) or Spanish SEProt (<http://www2.cbm.uam.es/~seprot/>), room for non-  
105 human proteomics and groups interested in different type of organisms (microorganisms, plant, and

animals) has been recently created, or is under discussion. The vision or sensations at the plant specific organization INPPO is that we are surviving within the field as a scientific ghetto. With no doubt, plant and human research can and should benefit from each other. Plants provide an almost unlimited experimental system and despite being far in evolution they share an important number of genes [10], with similar mechanisms of, for example, immunity [11], and host-pathogen interactions [12]. In addition, plants may be sources of drugs for disease treatment [13] and also of allergens [14, 15]. Some more examples of common phenomena between plant and animals are totipotency, the property of the cells to be developmentally reprogrammed, the conceptual basis of the stem cell projects [16], and the involvement of telomeres and telomerases in senescence and aging [17].

The topic of plant proteomics continues to be extensively reviewed, reflecting its expansion, relevance, and expectations; although we should assume by now that there is a long distance between expectations and realities, and that the full potential of proteomics is far, once again if compared with humans, from being fully exploited in plant biology research and crop science [18-20]. Here, in *Proteomics*, and also in other journals, much attention has been given to proteomics of plant stresses responses, the major topic in plant biology because of its implication in crop protection and biotechnological improvement [21-23]. Up to 15 plant proteomics reviews have been recently published in *Proteomics*, most of them in the Vol. 15, Iss. 5-6 (Reviews 2015) and 10 (Plant-based Foods: Seed, Nutrition and Human Health). Strikingly, the thematic issues covering the most recent technical novelties or the frontier in proteomics and biology knowledge have none or at most two plant papers per issue; it was the case of *Tissue Proteomics and Imaging Mass Spectrometry* (Vol.14, Iss. 7-8, one plant reference by Maciel et al., 2014 [24]), and *OMICS Views on Protein N-terminal Biology* (Vol. 15, Iss. 4; two plant original papers by Zhang et al., 2015 [25] and Venne et al., 2015 [26]). The review by Walton et al. (2015) [27] on signaling, a topic later discussed, deserves special attention, and is one of the plant reviews we should not miss. Regarding plant reviews in other journals, at least 15 have appeared, with some of them covering topics that will be mentioned later, including software, database and repositories [28] PTMs [29, 30], and signaling [31].

Accepted Article

132 Recently, Jorin-Novo (2015) [32] published an opinion article in which scientific standards  
133 and MIAPE were discussed in the context of proteomics work and publications. From his experience,  
134 the failure to meet proper scientific standards and those requested for a proteomics paper is the most  
135 obvious and immediate reason for rejection. As just a simple example, the general terminology  
136 employed in the literature included terms such as protein expression, when proteomics approaches  
137 actually determine abundance [1, 32]. Abundance is the result not only of gene expression  
138 (transcription and translation) but also of protein degradation and PTMs.

139 The list of referenced papers did not pretend to be exhaustive, but limited, according to the  
140 author's considerations, to those that presented some methodological, biological or translational  
141 novelties. Some of the revised papers fit in the general group of comparative analysis based on 2-DE  
142 coupled to MS analysis and they were not included. On the other hand, special attention was given to  
143 those using second to fourth generation techniques (labelling, gel-free, SRM/MRM [1]), those  
144 integrating classic and -omics approaches, or focused on PTMs and interactomics. Regarding the  
145 previous period reviewed, there have not been real novelties in the experimental (plant) system used,  
146 the biological process studied or the translational purpose pursued [1]. There is still a general need to  
147 go beyond the construction of a protein catalog accompanied by more speculation than real  
148 discussion, although better annotations and deeper knowledge is required in some cases.

## 150 WET AND DRY METHODOLOGIES

### 151 *In vitro* and *in situ* proteomics analysis

152 Proteomics is a discipline in expansion with continuous novelties and improvements in  
153 techniques, protocols, softwares and equipments [33, 34]. This is also true for quite standard and well  
154 established protocols in the early proteomics workflow stages prior to MS analysis, as it is important to  
155 optimize them to each experimental system (plant, organ, tissue, cell or subcellular fraction). Thus,  
156 improved protocols dealing with protein extraction [35], depletion [36, 37], fractionation [38], gel

staining [39], multiplexing [40], trypsin protein digestion [41] and sample preparation for shotgun analysis [42, 43] have recently been published.

The use of different MS platforms in plant proteomics analysis was previously discussed [1], however it is important to mention that new ion fragmentation procedures as alternative to the current collision induced dissociation, CID, and electron transfer dissociation, ETD, such as photodissociation, have been claimed to produce enhanced sequence coverage and higher confidence in sequence assignment [44, 45].

One of the limitations of proteomics, when compared to genomic and transcriptomic analyses, is that proteins cannot be amplified as can DNA and therefore, optimized and efficient extraction methods must be developed. Thus, the very laborious and time consuming used protocols and the number of replicates (the three biological replicates performed as a rule are not enough to perform solid statistical analysis and get confident biological conclusions [46], it is recommended to perform at least 5 biological replicates to allow a reliable estimation of residual variance when more than two treatments are compared) are the main bottleneck that makes proteomics unpopular among plant biologists. The use of an appropriate extraction method is even more relevant for PTMs or interactomics studies that request fractionation steps. These issues have been addressed by Krahmer et al (2015) [47], who compared different alternatives for sample preparation compatible with large numbers of samples in phosphoproteomics analysis.

Protein extraction is still challenging and constitutes a big difficulty in the analysis of specific proteins such as integral membrane proteins [48]. Integral membrane proteomics analyses involve complicated protocols that include membrane purification and protein extraction. In order to analyze the symbiosome integral membrane proteins, Clarke et al. (2015) [49] reported a protocol that included isolation of symbiosomes with three-step Percoll gradients and preparation of the membrane system by ultracentrifugation of the broken symbiosomes supernatant, followed by bicarbonate stripping and chloroform-methanol protein extraction prior to MS analysis. Although the excessive manipulation of the sample may interfere in the integral membrane protein analysis it has a great



value from a descriptive point of view. Once the protein is identified, the expression of the corresponding genes and the associated phenotype can be assessed by using complementary approaches. As an example, in order to understand intracellular protein trafficking (a hot topic in plant biochemistry), Heard et al. (2014) [50] enriched partially overlapping subsamples of endosomal proteomes associated with endomembrane from *A. thaliana* seedlings and used affinity purification of fluorescent fusion constructs for seven subcellular marker proteins in order to predict its precise or shared location, a strategy that resembles LOPIT [51]

From the current literature it is clear that plant proteomics is moving from 2-DE/MS (still the bases of an important number of works published) to shotgun (bottom up gel free label free). Shotgun will be the dominant platform in the near future if accompanied by a powerful and potent MS analysis and equipment. It is simple and does not require much manipulation of the sample. The iTRAQ, quite popular among the Chinese groups, appeared as the third alternative, even though plant proteomicists did not pay much attention in the past to labelling techniques [52-55]. By using this technique, Pan et al. (2015) [56] have reported up to 13,706 and 12,124 proteins identified with a LTQ-Orbitrap hybrid mass spectrometer in *Arabidopsis thaliana* roots and leaves. Changes in abundance between plants supplied with different iron concentrations were observed for 886 proteins with only a low percentage of them showing good correlation with transcript levels.

Whatever the employed platform, it is very important that it is validated, as a preliminary step, for the experimental system being analyzed, as for any analytical tool, in order to allow the determination of the number of proteins confidently identified and quantified. Serial dilution of the sample could be enough for validation purposes for those approaches based on label-free [57].

Fourth generation *in vitro* proteomics, SRM, MRM, or mass-western, sustain hypothesis driven protein analysis. In the surveyed period just one paper, by Rogniaux et al. (2015) [58] was published in *Proteomics* reporting the use of targeted approach for determining the relative abundance of allergens in several wheat varieties, and showing the potential of the technique for translational, and food traceability, purposes. Although the use of these techniques in plants is still limited, targeted

proteomics is the way to go for validation of the candidate proteins identified in 2-DE or shotgun analyses. It is certainly a more appropriate follow-up study when compared to qRT-PCR analysis frequently reported in proteomics publications.

Imaging has opened new possibilities for *in vivo* or *in situ* proteomics analysis and integration with cellular biology techniques [59], giving, at the same time, the possibility of proteomics analysis at the cellular level [60]. It is a more or less well established technique for metabolomics analysis [61] but under development for proteins [62] and plant tissue samples. The technique has been the focus of the *Proteomics Tissue Proteomics and Imaging Mass Spectrometry* special issue (Vol. 14, Iss. 7-8), in which, two plant studies were reported. Maciel et al. (2014) [24] used the laser ablation imaging technique to evaluate the distribution of selenium and sulfur in leaves of transgenic and non-transgenic *A. thaliana*. One of the limitations of imaging studies is the processing of high amounts of datasets, and therefore attention has been given to data processing methods, as discussed by Wijetunge et al. [63] and Franceschi and Wehrens (2014) [64].

#### ***In silico* analysis and data interpretation: software, databases and repositories**

Proteomics, as any -omics approach, generates from hundreds to thousands of identified products, being impossible to analyze and interpret all the data in a classic, mind based, manner [65]. The high amount of data generated and the costs of the analysis are the main reasons for the low number of samples and biological replicates analyzed. Protein profiling requires software and algorithms for protein identification, quantitation, functional and structural characterization, location, interaction, networks, integration, visualization and deposition in databases and repositories. These softwares are continuously created, improved and updated [66-69]. Recently, Sakata and Komatsu (2014) [28] have surveyed and reviewed plant proteomics databases and websites as well as recent technologies related to data collection and annotation.

The use of most algorithms and softwares is far from the current capacity of most plant biochemists working with proteomics. This is the reason why the dry part of the work is in the hands

of mass spectrometrists or bioinformaticians usually with no expertise in plant biology. This results in, more often than would be advisable, a blind acceptance of the computer provided data without a minimum rationale and critical evaluation of the data confidence, making it difficult to conclude from a biological point of view. The quite dynamic character of the proteomes and the fixed film provided by our experiments makes most interpretations speculative. The situation for a standard plant biochemist is even worse considering the number of algorithms that is continuously appearing [70]. Which one, what for, how to use them and translate to a biological discussion could be hell for most of us [71, 72]. In this regard, *Proteomics* a special issue, *Proteomics Data Visualization* (17, 10). In this and other regular issues a number of reviews discussing these questions have appeared [73-78]

The integration of disseminated data through databases is a pursued objective that will benefit the scientific community. In this direction, The BioMart Community Portal ([www.biomart.org](http://www.biomart.org)) is a good example the plant community should follow [79]. It includes over 800 different biological datasets spanning genomics, proteomics, model organisms, cancer data, ontology information and more.

## PROTEOMICS INTEGRATION WITH OTHER -OMICS

Within the -omics approaches, the advantage, if so, of proteomics, and also metabolomics, is that the information provided is closer to the phenotype [80]. On the other hand, it is much more complicate at the methodological level (i.e. genomics and transcriptomics are favored by PCR and well established microarray techniques). At the molecular level, the phenotype is the result of gene expression through transcriptional and translational events and gene products and metabolites interaction, so proteins are just part of the story. As previously discussed [1], proteomics has limitations, the more relevant being proteome coverage, quantitation, identification in orphan organisms, PTM, and interactomics. Protein identification and quantification cannot always be as confident as we wish because of the organism, experimental design, and the employed techniques and

261 protocols, making our data interpretation conservative or speculative. In comparative proteomics, the  
262 high variability in the protein profile among biological samples and the low number of biological  
263 replicates employed in most studies hinder the biological interpretation. Thus, it is not easy to establish  
264 protein abundance ranges in normal (control) samples, as it has been shown by Higdon et al. (2015)  
265 [81]. These authors used data from two human proteome studies featured in Nature, and found that  
266 protein abundance among biological replicates varied by  $\pm 4$ - to 10-fold for most proteins, with  
267 coefficient of variation (CV) in between 62 and 117 %. To control or reduce this CV, it is  
268 recommended to compare proteins identified with at least 3 or more unique peptides and normalize  
269 abundances against housekeeping proteins with low variability.

270 Just by itself, and more than generating a list or catalog of protein species, proteomics can  
271 provide real biological knowledge; however it would require, first, the use of a model systems, in  
272 plants mostly *A. thaliana*, with a good collection of mutants for reverse and functional genetics  
273 studies and, second, an appropriate experimental system, as for example cell suspensions. A good  
274 example of this is the paper published by Smith et al. (2015) [82] in which signal transduction  
275 pathways mediating plant programmed cell death was studied. By using isobaric-tagged relative and  
276 absolute quantitation of proteins present in the extracellular matrix, 33 proteins were identified as  
277 putative cell-death regulatory proteins. One of these proteins, the CYCLASE 1, which had previously  
278 no known function, was a negative regulator of cell death in *Arabidopsis*.

279 The integration of the different -omics, in the direction of systems biology, is far from being a  
280 reality or was only possible for a reduced number of research groups or through large international  
281 consortia [83]. Systems biology, wide genomic approaches, and the recently coined proteogenomics,  
282 in combination with image phenotyping, classical molecular, physiological and cell biology  
283 approaches [84, 85] will get us to a deeper and better rather than to a partial and biased knowledge of  
284 the biological systems. This knowledge is highly relevant for crop improvement aiming at  
285 productivity yield increases and survival under extreme environmental conditions through classical,  
286 molecular marker assisted, and biotechnological breeding [11, 86-88]

Combined proteomics and transcriptomics analysis has been used in the study of the maize response to waterlogging and resulted, in general, in good correlations between both data [54]. But this is not necessarily the rule and it depends on the gene or gene groups, being the consequence of transcriptional, posttranscriptional, translational and posttranslational events. Abundance of both molecules depends not only on their synthesis but also on their stability and degradation. It is also known that different mRNAs possess different translational efficacy [89].

At least one paper, by Gupta et al. (2015) [90], has been published in which integrated proteomics and metabolomics have been employed as experimental approaches. They analyzed differences between soybean lines with different color in the seed coat. Differences in flavonoids, which provide the molecular basis of the color, were correlated with levels in pathway-related enzymes. Finally, there are not many papers in which the combined use of the three -omics approaches are reported. In the surveyed period, two papers dealt with the detection of unintended effects in transgenic maize [91, 92].

In any case, single and easy multi-omics approach experiments can be always performed and the preliminary steps of the workflow should be evaluated as one of the limitations is just preparative. In order to make confident data comparison or validation, these preparations should come from the same sample. Independent experiments or extractions can originate artefacts or biases, mostly coming from the inherent biological variability. In this direction, recent workflows aimed to extract biomolecules (DNA, RNAs, proteins, and metabolites) have been developed [93]. Despite dedicated workflows showed their efficiency, combined protocols demonstrated their performance in isolating biomolecules suitable to be processed by high-throughput pipelines (gel free label free proteomics, high-throughput metabolomics analyses and next generation sequencing transcriptomic analyses of messenger or regulatory RNAs) while avoiding biases related to the use of not exactly the same sample for the different -omics level analyses. The full overlapping of the datasets makes it easier to interpret and analyze the data when performing a Systems Biology-based integrative approach.

Proteogenomics is a relatively new and emerging field at the intersection of genomics and proteomics, being part of the wide genomics approaches. Although it may have multiple derivations (RNA and protein correlation studies, proteomics and data validation, gene sequence analysis, among others), in the simplest way it can be understood as the use of proteomics data and protein MS derived sequences to improve gene sequence annotations [94-96]. From the proteomics perspective it has an important feedback as correct sequence annotation will increase the number and confidence of the protein identification [97], favoring, at the same time the studies of non-model organisms [98]. It will also go deeper into the knowledge of posttranscriptional and translational events giving rise to the different gene products, protein species or proteoforms [99].

The topic has been deeply reviewed in *Proteomics* (*Proteogenomics* special issue; Vol. 14, Iss. 23-24). Proteogenomic experiments and prediction tools will differ between genome-sequenced and –unsequenced, as well as eukaryotic or prokaryotic organisms [95, 100].

## FROM PROTEOMICS TO BIOLOGY

Cell communication, receptors, and signaling pathways, are good examples of how proteomics can contribute to biological knowledge and be used for translational purposes. This topic has been well presented in a recent, excellent review, by Walton et al. (2015) [27]. Signaling is a hot topic of great relevance in biology mainly approached by using functional and reverse genetics. It is quite challenging as it deals with minor and recalcitrant, membrane bound, proteins, PTMs and interactions, whose investigation requires integration with classic, transcriptomics and metabolomics approaches. In the recent literature surveyed in the reviewed period, this topic is well covered with both review papers and original contributions. Some other topics are also of interest but are somehow related to this or are less represented in the most recent literature. This is the case of plant developmental processes, plant-pathogen interactions and responses to other biotic stresses [101-104].

Plants chemically communicate and interact with their biotic and abiotic surroundings, responding and adapting its biology to it. They also complete their developmental program (from seed

germination to seed production through vegetative and reproductive phases) in coordination with seasonal changes noticed mainly as variations in light, temperature and water availability. There are also daily changes associated to a circadian clock timed by, among other factors, light regimes. The integration of environmental responses with development is crucial for plant survival, growth, and from an agronomic point of view, productivity. Plants, as sessile organisms, are very versatile, and their genome expression and metabolism is continuously reprogrammed in response to physico-chemical and biological stimuli. Despite the high number of specialized organs, tissues and cell types within a plant, all of them respond coordinately, implicating in good intercellular connexion systems with chemical messages between receptor and effector cells. At the individual cell level, gene expression changes are mediated by receptors, intracellular and interorganule signals, and signal transduction pathways resulting in changes in gene expression through transcriptional, translational and posttranslational events. Quite a number of molecules are known that act as external or internal chemical signals, including phytohormones, and those from microorganisms (symbionts, parasites or pathogens) [101, 103-106]. On the other side, receptors and signal transduction pathways and their components are starting to be characterized [31, 107].

Proteomics is contributing to the above processes through descriptive, comparative, PTMs, and interaction studies. Walton et al. (2015) [26] updated the contribution in these areas and the different strategies employed in dissecting plant hormone signaling. Some following papers dealing with PTMs and interactomics related to cell signaling are highlighted. Others, using descriptive or comparative proteomics approaches have been skipped from this review.

Protein phosphorylation is the most common mechanism of signal and because of that phosphoproteomics is the immediate strategy to be employed. Silva-Sanchez et al., (2015) [108], and Li et al., (2015) [29] have summarized the current methodologies used in extracting, enriching, and fractionating phosphoproteins, and in their MS analyses for identification, quantitation, and phosphorylation site mapping, conclude on cross-talk between different PTMs (phosphorylation, glycosylation, and redox modifications) and data validation. They provided a list of phosphoproteins,

softwares and databases. As an alternative to the current protocols, Han et al. (2015) [109] have proposed a faster workflow analysis in the case of sample amount limitation that increases coverage; it included integrated multistep enzymatic digestion, phosphopeptide enrichment and database search (iMEED). In an attempt to elucidate post-translational processes mediating circadian events, Choudhary et al. (2015) [110] performed a phosphoprotein analysis employing TiO<sub>2</sub> phosphopeptide and MS analysis. They identified 1586 phosphopeptides on 1080 protein groups, out of which 102 changed in abundance, including clock proteins (ELF4, PRR3), transcription factors and kinases. By using isotope-assisted quantitative phosphoproteomics, Minkoff et al. (2015) [111] carried out a comparative phosphoproteomics study between wild and mutant ABA-insensitive *A. thaliana* plants. Multiple changes occurred as early as 5 minutes after ABA treatment, with a reduction of the phosphorylation events in the mutant.

Reactive oxygen species overproduction is a typical plant response to oxidative stress, these species acting as signals and causing protein redox state changes [30, 112, 113]. The reversible PTM methionine oxidation was studied in *A. thaliana* plants by Jacques et al. (2015) [114] using wild and catalase-2 knock-out mutants. Over 500 sites of oxidation in 400 proteins were reported. They showed that the activity of two specific glutathione-S-transferases was significantly reduced upon oxidation. Konert et al. (2015) [115] have gone deeper into the mechanism of ROS signaling and metabolic adjustment in *A. thaliana*. By using genetic, biochemical, and a combination of MS data-dependent acquisition and SRM, they showed how protein phosphatase 2A, through its regulatory B'  $\gamma$  subunit, interact with a cytosolic aconitase and control mitochondrial alternative oxidases.

Interactomics is the most challenging and less developed proteomics approach, but the most concluding one in order to probe *in vitro* and *in situ* better than *in silico* interactions between proteins and proteins and DNA, RNA or metabolites. It will help in developing models from networks mediating receptor capture of signals and gene expression changes. The standard proteomics analysis involves the capture of protein partners (affinity purification or immunoprecipitation) coupled to MS analysis. Other published approaches included *in vivo* cross-linking with para-formaldehyde and



immunoaffinity purification of cross-linked protein. It has been used by Obermeyer et al., (2014) [116] in the search for H(+) ATPase complexes in lily pollen. These techniques require high amount of sample and involve excessive manipulation, being subjected to biases or false positives. Microarray technology will be, with no doubt, the future for more feasible experiments in which dynamic interactions could be observed through time course experiments [117]. As an alternative, the parallel analysis of genomic, transcriptomics, and proteomics experiments could be performed [118].

### Some missed relevant papers

Last but not least in the surveyed period are the publications covering topics that deserve to be mentioned in a general plant proteomics review, either because they report on technical innovations or novel applications to the plant biology research. As we did not intend to be exhaustive, and due to space limitation, we will just mention them.

Such is the case of the top-down proteomics [45, 119-121], N-acetylome [122], degradation of proteins with N-termini containing basic amino acids (the Arg/N-end rule) [25], and single cell type proteomics analysis. Plant organs are complex in terms of the different types of tissues and cells that are formed, with each one having its own proteome signature. Thus, for comparative proteomics purposes it is possible that only a discrete number of cells respond to the experimental variable under study and if so, by extracting the whole organ we might be diluting the response. In order to decipher single proteome analysis new methodologies should be developed [86]. Up to now the laser microdissection [123] has been applied, however it has been of very limited use with plants. What is close to be a technical reality for transcriptomics looks impossible for proteomics [124].

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